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(54) Title: EXPRESSION OF HUMAN CMV GLYCOPROTEIN-H USING THE BACULOVIRUS-INSECT CELL EXPRESSION SYSTEM

(57) Abstract

Methods for producing recombinant polypeptides which are immunological equivalents of human cytomegalovirus glycoprotein H are disclosed. The methods involve the modification of native CMV gH by C-terminal truncation and subsequent expression in baculovirus/insect cell expression systems. Also disclosed are vectors, recombinant cells and modified recombinant glycoproteins.

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EXPRESSION OF HUMAN CMV GLYCOPROTEIN-H USING THE BACULOVIRUS-INSECT CELL EXPRESSION SYSTEM

Background of the Invention

Technical Field

This invention relates generally to vaccines and diagnostics. More particularly it relates to methods of producing recombinant human CMV glycoproteins, particularly, to the use of the baculovirus-insect cell expression system to express useful amounts of glycoprotein-H (gH).

Description of Related Art

Human cytomegalovirus (CMV) is a ubiquitous agent in human populations. Infections are generally asymptomatic, but there can be serious medical manifestations of the disease in immunocompromised individuals (transplant recipients and AIDS patients) and in congenitally infected newborns.

While human strains of CMV have been tested in vaccines, there are many objections to the use of experimental live attenuated virus vaccines. In the absence of a complete understanding of the biology of CMV, the most rational approach to a vaccine would involve the development of subunit vaccines based upon the surface glycoproteins of the virus using recombinant viral glycoproteins which elicit neutralizing antibodies. Unfortunately, most recombinant approaches to this

problem have not provided large amounts of glycoprotein for use in vaccines.

CMV specifies multiple glycoproteins (Stinski, M. (1976) J Virol 19:924-932; Pereira, L., et al. (1982) Infect Immun 36:924-932). Characterization of these has involved studies of CMV-infected cells and purified virions using polyclonal and monoclonal antibodies (Pereira, L., et al. (1984) Virology 139:73-86; Britt, W.J., (1984) Virology 135:369-378; Nowak, B., et al. (1984) Virology 132:325-338; Law, K.M., et al. (1985) J Med Virol 17:255-266; Rasmussen, L., et al. (1984) Proc Natl Acad Sci USA 81:876-880; and Britt and Auger (1986) J Virol 58:185-191).

Among the polypeptides which have been associated with human CMV is an 86 kD glycoprotein (gH) which has been identified in CMV-infected cells and in virions and shown to induce complement independent neutralizing antibodies in guinea pigs (Rasmussen et al., supra, and (1985) Virology 145:186-190; Pachl, C. et al., Virology (1989) 169:418-426; USSN 367,363).

Cranage et al. (1988) J Virol 62:1416-1422 reported that mapping and sequence analysis of the CMV genome indicated that one fragment, designated HindIII L, contained an open reading frame showing some similarity to the gH genes of herpes simplex virus 1 (HSV1) and Epstein-Barr virus (EBV). This report also showed that the 3' end of the CMV gH gene was located 222 bp from a HindIII site at the boundary of the HindIII L fragment and the immediately upstream HindIII fragment, designated HindIII D. This report is reiterated in PCT/GB87/00164 (pub. no. WO87/05326). That PCT application further describes the cloning and structure of the human CMV gH gene and its expression using vaccinia virus.

The applicants teach, in the commonly assigned copending U.S.S.N. 367,363, filed 16 June 1989, herein incorporated by reference, the location and sequence of a structural gene of human CMV which encodes gH, together with the gH amino acid sequence. The gene and various truncated analogs thereof were inserted into mammalian expression vectors and recombinant human CMV gH and truncated analogs thereof were expressed at low but detectable levels in COS cells. The recombinant gH and certain analogs were immunologically reactive with a neutralizing monoclonal antibody (murine monoclonal 1G6, Rasmussen et al, PNAS (1984) 81:876-880) to the native protein (gp86).

In spite of the progress which has been made, a major obstacle to the development of a CMV subunit vaccine has been the inability to express high levels of human CMV gH-related polypeptides in any of the conventional expression systems.

Recently, an expression system has been developed which utilizes baculovirus vectors to introduce mammalian structural genes into insect cells in culture and subsequently effect the expression of the heterologous polypeptide. This has proven successful for the recombinant expression of some proteins. See for example, Ju, G., et al., Curr. Communic. in Mol. Biol. - Gene Transfer vectors for Mammalian Cells (1987) C.S.H.L. Press pps. 39-45); Atkinson, A.E., et al., Pestic. Sci. (1990) 28:215-224.

In some instances, viral proteins were expressed. See for example, PCT Pub. No. WO90/02566 (paramyxovirus fusion protein); EPO Pub. No. 341,611 (canine parvovirus Vp-2); EPO Pub. No. 329,257 (plasmodium circumsporozoite antigen); PCT Pub. No. WO89/05823 (chimeric human respiratory syncytial viral

antigen); and EPO Pub. No. 272,858 (HIV envelope protein).

In some instances, subunit proteins of viruses in the Herpes family have been disclosed. See for example, Frech, B. et al., J. Virol. (1990) 64:2759-2767 (EBV TP-1); Kristie, T.M., et al., EMBO J. (1989) 8:4229-4238(HSV alpha TIF); Dodson, M.S., et al., J. Biol. Chem. (1989) 264:20835-20838 (HSV-1 helicase); Olivo, P.D., et al., J. Virol. (1989) 63:196-204 (HSV-1 replication enzymes); PCT Pub. No. WO89/10965 (pseudo-rabies and infectious bovine rhinotracheitus virus polypeptides); and EPO Pub. No. 340,359 (CMV immediate early protein).

Removal of the transmembrane domain of the viral cell surface glycoproteins of influenza virus (Sveda, M.M., et al., Cell (1982) 30:649-656; Gething, M.J. & J. Sambrook, Nature (1982) 300:598-603) and vesicular stomatitis virus (Rose, J.K. & J. E. Bergmann, Cell (1982) 30:753-762) results in secretion of the truncated glycoprotein from mammalian host cells. See also EPO Pub. No. 139,417. As noted above, such an approach for gH expression in mammalian cells has not produced acceptable levels of expression.

Disclosure of the Invention

Summary of the Invention

A general difficulty of mammalian recombinant gene expression is that many proteins are resistive to expression, particularly high levels of expression, in many systems and the likelihood of success for expression of any given protein is difficult to predict.

Previous attempts to express high levels of human CMV gH have not been successful. Consequently applicants attempted to express gH in the baculovirus/

insect cell expression system. Surprisingly, it was found that at least a 10 fold increase (relative to expression in mammalian cells) in the expression of an immunologically useful gH polypeptide can be achieved.

The subject invention discloses materials and methods for producing a human CMV polypeptide, particularly gH analogues, by means of: a) providing an insect host cell which comprises DNA encoding the polypeptide; b) incubating the cell under conditions in which the polypeptide is expressed; and c) isolating the polypeptide.

In one embodiment the invention includes baculovirus transfer vectors which consist of a bacterial plasmid which comprises DNA which is substantially homologous to baculovirus DNA, and a segment of DNA which encodes a CMV gH polypeptide.

In another embodiment the invention includes a baculovirus comprising DNA which encodes a human CMV gH polypeptide in which the expression of the polypeptide is controlled by a baculovirus regulatory element.

Another embodiment is an insect cell containing DNA which encodes a human CMV gH polypeptide which is capable of expressing the polypeptide in a useful amount.

In a final embodiment the invention includes a CMV gH polypeptide which is recognized by an antibody with specificity for CMV gH wherein the polypeptide is produced by the method disclosed below.

Brief Description of the Drawings

FIG. 1 depicts restriction maps showing the position of the gH gene within the CMV Towne genome. Line A is a HindIII restriction map of the 235 kb CMV Towne genome displayed in the prototype orientation. Unique sequences are indicated by a thin line and the

repeated elements ab, b'a'c', ca are denoted by boxes. Line B is the restriction map of the 3910 bp HindIII A/H to PstI fragment encoding gH. The black bar corresponds to the gH coding region and the arrow indicates the direction of transcription. Restriction site designations are: A, ApaI; B, BamHI; Bg, BglII; Bs, BsphI; E, EcoRI; H, HindIII; P, PstI; S, SmaI; T, Tth111I.

FIG. 2 is the nucleotide sequence of the CMV Towne gH gene. The DNA sequence and the predicted amino acid sequence are shown. The putative TATA, CAT and polyadenylation sequences have been underlined. Potential N-linked glycosylation sites are overlined and the predicted signal sequence and transmembrane domain are boxed. The locations of p86 tryptic peptides are indicated by the broken lines (Gln_{354} to Pro_{364} and Gln_{370} to Gln_{377}).

FIG. 3 is a chart of the hydropathic analysis of the CMV Towne gH. The gH protein is displayed from Met_1 to Cys_{742}, left to right, along the x-axis. The relative hydropathy at each position is computed using a moving window of seven amino acids. The points above the x-axis indicate increasing hydrophilicity and points below the axis indicate increasing hydrophobicity.

FIG. 4 is a diagram of the bacterial plasmid pAc373, commonly used as a transfer vector in the baculovirus (AcNPV) expression system showing restriction endonuclease sites. The bold portion of the plasmid depicts the baculovirus sequences with the polyhedrin gene shown as an open bar. The nucleotide and amino acid sequences around the start codon of the polyhedrin gene are also shown."+1" corresponds to the "A" of the AUG start codon.

FIG. 5 is a diagram of a gH (pACgH2) and truncated gH (pACgH6) transfer vector in which the CMV gH coding sequences have been spliced into the BamH1 site of pAc373.

FIG. 6 shows the secretion of truncated gH from insect cells infected with a baculovirus recombinant expressing truncated gH.

FIG. 7 shows the expression over time of secreted gH and truncated gH from Sf9 cells infected with full-length or C-terminal truncated baculovirus recombinants as measured by ELISA assay.

FIG. 8 shows the secretion of truncated gH expressed by mammalian CHO cells transfected with a mammalian cell expression plasmid, pCMAd-H6, and insect Sf9 cells infected with a baculovirus recombinant, pACgH6.

Detailed Description of the Invention

I. Definitions

"CMV gH polypeptide" refers to polypeptides comprising a fragment of native human CMV gH. Thus, the term includes both polypeptides comprising the native sequence of gH (full-length and truncated), as well as analogues thereof. Preferred analogues are those which are substantially homologous to the corresponding native amino acid sequence, and most preferably encode at least one native gH epitope, such as a neutralizing epitope. A particularly preferred class of CMV gH polypeptides are those lacking a sufficient portion of the C-terminal transmembrane domain to promote efficient expression and/or secretion of the CMV gH polypeptide at high levels from the insect cell expression hosts of the present invention. It is believed that the about 25 C-terminal amino acid residues (residues 717 to 742 of strain Towne,

FIG. 2) comprise the transmembrane domain, but other regions may also be critical to transmembrane binding. Deletions of all or parts of such domains that eliminate or substantially decrease transmembrane binding are desired, and typically will constitute a deletion of at least about 5 amino acids, preferably at least about 10 residues, and most preferably at least about 20 residues from the native domain sequence. Examples of such deletions from one strain are the residues numbered in Figure 2, 732 to 742, 722 to 742, 720 to 742, 717 to 742, and 712 to 742. Of course, other functional deletions can be readily defined by those of ordinary skill by constructing and screening deletions from the same or other domains by expressing the polypeptides in insect cells. The only true upper limit to the deletions is the practical limitations of retaining useful epitopes (e.g., neutralizing epitopes. Typically, however, the deletions will not constitute more than about 100 amino acids of the native gH sequence, particularly the 100 C-terminal residues. It should also be understood that "deletion" of a portion of a transmembrane domain means only that the particular native amino acid sequences do not appear in the polypeptide, and that other amino acids (such as hydrophilic residues) can be substituted for the deleted residues. The human CMV gH polypeptides of the present invention are produced in insect cell expression hosts. Thus, when the polypeptide has been secreted and glycosylated, it will have the glycosylation pattern unique to the expression of this polypeptide in an insect cell host.

The term "effective amount" refers to an amount of CMV gH polypeptide sufficient to induce an immune response in the subject to which it is administered. The immune response may comprise, without limitation,

induction of cellular and/or humoral immunity. Preferably, the effective amount is sufficient to effect treatment, as defined above. The exact amount necessary will vary from subject to subject depending on the species, age, and general condition of the subject, the severity of the condition being treated, the particular polypeptide selected, its mode of administration, etc. Thus, it is not possible to specify an exact effective amount. However, the appropriate effective amount may be determined by one of ordinary skill in the art using only routine experimentation.

A "fragment" of a reference polypeptide is any contiguous amino acid sequence found in the reference polypeptide. Preferably, the fragment encodes an epitope from the polypeptide, most preferably a neutralizing epitope. A first polypeptide comprises a fragment of another polypeptide even if the homologous domain in the first polypeptide is flanked by amino acid sequences which are not fragments of the other polypeptide.

A polypeptide is "immunologically reactive" with an antibody when it is capable of being specifically recognized and bound by an antibody. Immunological reactivity may be determined in a standard immunoassay, such as a competition assay, as is known in the art.

"Operably linked" refers to a juxtaposition wherein the components so described are in a relationship permitting them to function in their intended manner. A regulatory element "operably linked" to a structural sequence is ligated in such a way that expression of the structural sequence is achieved under conditions compatible with the regulatory elements.

The term "polypeptide" refers to a polymer of amino acid residues and is not limited to a minimum length of the product. Thus, peptides, oligopeptides,

and proteins are included within the definition of polypeptide. This term also includes post-expression modifications of the polypeptide, for example, glycosylation, acetylation, phosphorylation and the like.

"Recombinant" as used herein to describe a polynucleotide means a polynucleotide of genomic, cDNA, semisynthetic, or synthetic origin which, by virtue of its origin or manipulation: (1) is not associated with all or a portion of the polynucleotide with which it is associated in nature; and/or (2) is linked to a polynucleotide other than that to which it is linked in nature. The term "recombinant" as used with respect to a protein or polypeptide means a polypeptide produced by expression of a recombinant polynucleotide. "Recombinant host cells", "host cells", "cells", "cell lines", "cell cultures", and other such terms denoting prokaryotic microorganisms or eukaryotic cell lines cultured as unicellular entities, are used interchangeably, and refer to cells which can be, or have been, used as recipients for recombinant vector or other transfer DNA, and include the progeny of the original cell which has been transfected. It is understood that the progeny of a single parental cell may not necessarily be completely identical in morphology or in genomic or total DNA complement to the original parent, due to accidental or deliberate mutation. Progeny of the parental cell which are sufficiently similar to the parent to be characterized by the relevant property, such as the presence of a nucleotide sequence encoding a desired peptide, are included in the progeny intended by this definition, and are covered by the above terms.

A "regulatory element" refers to a polynucleotide sequence which effects the expression of a coding sequence to which it is linked. The nature of

such regulatory elements in eukaryotes, particularly insect cells, include promoters, terminators, leader sequences and, in some instances, enhancers.

A "replicon" is any genetic element, e.g., plasmid, cosmid, chromosome, virus, or phage, that behaves as an autonomous unit of polynucleotide replication within a cell.

A sequence which is "substantially homologous" to a reference sequence shares at least about 50% sequence homology, preferably at least about 75%, more preferably at least about 85%, and most preferably at least about 90%.

"Transformation", as used herein, refers to the insertion of an exogenous polynucleotide into a host cell, irrespective of the method used for insertion: for example, direct uptake, transduction, or f-mating. The exogenous polynucleotide may be maintained as a non-integrated vector, for example, a plasmid, or alternatively, may be integrated into the host genome.

As used herein, "treatment" refers to any of (i) the prevention of infection or reinfection as in a traditional vaccine, (ii) the reduction or elimination of symptoms, and (iii) the substantial elimination of the virus. Treatment may be effected prophylactically (by administration prior to infection) or therapeutically (by administration during or following infection).

A "vector" is a replicon in which a heterologous polynucleotide segment is attached, so as to bring about the replication and/or expression of the attached segment, such as a plasmid, transposon, phage, etc.

II. Modes of Carrying Out the Invention

A. Nucleotide and Amino Acid Sequencing of gH.

The two main laboratory isolates of human CMV are AD169 (Rasmussen, L. et al. Proc. Natl. Acad. Sci. USA (1984) 81:876-880), and strain Towne (Pachl, C. et al., Virology (1989) 169:418-426). Both strains encode a gH, and these glycoproteins share substantial sequence similarity and are immunological reactivity. Other strain;s of CMV can readily be used for the source of gH sequences.

The identification and isolation of the 3910 bp Hind III to Pst I fragment of the strain Towne CMV genome, which contains a 2226 bp gH open reading frame is described in Pachl, C. et al., Virology (1989) 169:418-426 herein incorporated by reference, and shown in Figures 1 & 2. The gH sequence is 742 amino acids (84.3 kD) and has the characteristics of a membrane glycoprotein. The hydropathic profile, Figure 3, indicates a hydrophobic N-terminal domain which is likely to be a cleavable signal sequence. As indicated in Figure 2, the predicted external hydrophilic region of gH, residues Arg_{24} to Arg_{717}, contained six possible sites for N-linked glycosylation, Asn-X-Thr/Ser, where X can be any of the 20 amino acids.

Immunologically equivalent fragments of human CMV gH, for example, from AD169 or strain Towne, may be identified by making analogs of the polynucleotide sequence encoding the protein that are truncated at the 3' and/or 5' ends of the sequence and/or have one or more internal deletions, expressing the analog polynucleotide sequences, and determining whether the resulting fragments immunologically react with a CMV antibody or induce the production of such antibodies in vivo, particularly neutralizing antibodies. For example,

deletion within or encompassing a 13 amino acid hydrophobic peptide (residues Met_{340} to Ala_{352}) may facilitate secretion of gH. Similarly, deletions from within or encompassing the 33 residues (709 -742) from the C-terminal transmembrane and internal regions facilitate secretion. Several different truncated versions of gH are described in USSN 367,363, including deletion of the last 22 C-terminal residues (equivalent to the fragment described in Example 2, below). This fragment retains the first three amino acids of the putative gH transmembrane domain.

B. Fusion Proteins

Human CMV gH polypeptides may be produced in the form of a fusion protein. If it is desired to express a mature protein, then the structural sequence coding for the heterologous protein is spliced before the start codon of the baculovirus polypeptide coding sequence, for example the start codon ATG of the polyhedrin gene, where A is nucleotide +1 in Figure 4. See Section II.C., below.

However, under some circumstances it may be desireable to retain the initial sequences of the baculoviral polypeptide in a fusion protein, for example to improve the efficiency of secretion in insect cells. Here the splicing sites for introduction of the heterologous coding sequence is downstream from the start codon of the viral gene. Vectors with unique transfer vector restriction sites from +5 to +175 of the polyhedrin gene are available from the laboratory of Max Summers (Summers & Smith, Texas Agricultural Experiment Station Bulletin No. 1555 (1987)).

It may also be desireable to express hybrid polypeptides which comprise CMV gH polypeptide, for

example, in order to improve expression, secretion, recovery or immunogenicity. These hybrids can be made by combining the DNA encoding a CMV gH polypeptide with DNA encoding an additional polypeptide fragment, for example a fragment related to hepatitis B surface antigen (HBSAg), beta-galactosidase, SOD, or ubiquitin, and expressing the combined DNA in the baculovirus/insect cell expression system. Hybrids may also sequences encoding epitopes from other CMV polypeptides, for example gB, or other viruses, for example HSV or HBV.

C. Baculovirus Expression Systems

The materials, methods and techniques used in constructing vectors, transfecting cells, picking plaques, growing cells in culture, and the like are known in the art and manuals are available describing these techniques. However, as a general guide the following sets forth the procedures, materials, and methods as well as commercial sources.

Generally, the components of the expression system include a transfer vector, usually a bacterial plasmid, which contains both a fragment of the baculovirus genome, and a convenient restriction site for insertion of the heterologous gene to be expressed; a wild type baculovirus with a sequences homologous to the baculovirus-specific fragment in the transfer vector (this allows for the homologous recombination of the heterologous gene in to the baculovirus genome); and appropriate insect host cells and growth media.

After inserting the heterologous gene into the transfer vector, the vector and the wild type viral genome are transfected into an insect host cell where the vector and viral genome recombine. The packaged recombinant virus is expressed and recombinant plaques

are identified and purified. Materials and methods for baculovirus/insect cell expression systems are commercially available in kit form from, inter alia, Invitrogen, San Diego CA. These techniques are generally known to those skilled in the art and fully described in Summers and Smith, Texas Agricultural Experiment Station Bulletin No. 1555 (1987), herein incorporated by reference.

C.1. - Vectors

Insect cells and compatible vectors which are useful as recombinant expression systems are known in the art, and include, for example, insect expression and transfer vectors derived from the baculovirus Autographa californica nuclear polyhedrosis virus (AcNPV), which is a helper-independent, viral expression vector. Viral expression vectors derived from this system usually use the strong viral polyhedrin gene promoter to drive expression of heterologous genes.

Prior to inserting the foreign gene into the baculovirus genome, the above described components, comprising a promoter, leader (if desired), coding sequence of interest, and transcription termination sequence, are typically assembled into an intermediate transplacement construct (transfer vector). Intermediate transplacement constructs are often maintained in a replicon, such as an extrachromosomal element (e.g., plasmids) capable of stable maintenance in a host, such as bacteria. The replicon will have a replication system, thus allowing it to be maintained in a suitable host for cloning and amplification.

Currently, the most commonly used transfer vector for introducing foreign genes into AcNPV is pAc373 (FIG. 4). Many other vectors, known to those of skill in

the art, have also been designed. These include, for example, pVL985 (which alters the polyhedrin start codon from ATG to ATT, and which introduces a BamHI cloning site 32 basepairs downstream from the ATT; See Luckow and Summers, Virology (1989) 17:31.

The AcNPV polyhedrin portion of transfer vectors pAc373 and pVL985, for expression of nonfused heterologous proteins, is shown in Figure 4. In the figure, the numbers shown refer to positions within the native gene, where the A of the ATG codon is +1. Figure 4 also shows a restriction endonuclease map of the transfer vector pAc373. The map shows that a unique BamHI site is located following position -8 with respect to the translation initiation codon ATG of the polyhedrin gene. There are no cleavage sites for SmaI, PstI, BglII, XbaI or SstI.

The plasmid usually also contains the polyhedrin polyadenylation signal (Miller et al. (1988) Ann. Rev. Microbiol., 42:177) and a procaryotic ampicillin-resistance (amp) gene and origin of replication for selection and propagation in E. coli.

After insertion of the heterologous gene, the transfer vector and wild type baculoviral genome are co-transfected into an insect cell host. The promoter and transcription termination sequence of the construct will typically comprise a 2-5kb section of the baculovirus genome. Methods for introducing heterologous DNA into the desired site in the baculovirus virus are known in the art. (See Summers and Smith (1987); Ju et al. (1987); Smith et al., Mol. Cell. Biol. (1983) 3:2156; and Luckow and Summers (1989)). For example, the insertion can be into a gene such as the polyhedrin gene, by homologous double crossover recombination; insertion can also be into a restriction enzyme site engineered into

the desired baculovirus gene. Miller et al., Bioessays (1989) 4:91.

The newly formed baculovirus expression vector is subsequently packaged into an infectious recombinant baculovirus and plaque purified by techniques known to those skilled in the art. Summers and Smith (1987); Miller et al. (1989).

C.2. Vector promoters

Baculovirus expression vectors usually contain a baculovirus promoter. A baculovirus promoter is any DNA sequence capable of binding a baculovirus RNA polymerase and initiating the downstream (3') transcription of a coding sequence (e.g. structural gene) into mRNA. A promoter will have a transcription initiation region which is usually placed proximal to the 5' end of the coding sequence. This transcription initiation region typically includes an RNA polymerase binding site and a transcription initiation site. A baculovirus promoter may also have a second domain called an enhancer, which, if present, is usually distal to the structural gene. Expression may be either regulated or constitutive.

Structural genes, abundantly transcribed at late times in the infection cycle, provide particularly useful promoter sequences. Examples include sequences derived from the gene encoding the viral polyhedron protein [Friesen et al. (1986) "The Regulation of Baculovirus Gene Expression," in: The Molecular Biology of Baculoviruses (ed. Walter Doerfler); E.P.O. Pub. Nos. 127,839 and 155,476] and the gene encoding the p10 protein [Vlak et al (1988) J. Gen. Virol. 69:765].

C.3. Intracellular Expression and Secretion

A recombinant polypeptide may be expressed intracellularly or, if it is expressed with the proper regulatory sequences, it can be secreted. Good intracellular expression of nonfused foreign proteins usually requires heterologous genes that ideally have a short leader sequence containing suitable translation initiation signals preceding an ATG start signal. If desired, methionine at the N-terminus may be cleaved from the mature protein by in vitro incubation with cyanogen bromide.

Alternatively, recombinant proteins can also be secreted from the host cell by creating chimeric DNA molecules that encode a fusion protein comprised of a leader sequence fragment that provides for secretion of the foreign protein in insects. The leader sequence fragment typically encodes a signal peptide comprised of hydrophobic amino acids which direct the secretion of the protein from the cell.

DNA encoding suitable signal sequences can be derived from genes for secreted insect or baculovirus proteins, such as the baculovirus polyhedrin gene (Carbonell et al. (1988) Gene, 73:409). Alternatively, since the signals for mammalian cell posttranslational modifications (such as signal peptide cleavage, proteolytic cleavage, and phosphorylation) appear to be recognized by insect cells, and the signals required for secretion and nuclear accumulation also appear to be conserved between the invertebrate cells and vertebrate cells, leaders of non-insect origin, such as those derived from genes encoding human α-interferon [Maeda et al. (1985) Nature, 315:592], human gastrin-releasing peptide [Lebacq-Verheyden et al. (1988) Molec. Cell. Biol., 8:3129], human IL-2 [Smith et al. (1985) Proc.

Natl. Acad. Sci. USA, 82:8404], mouse IL-3 [Miyajima et al. (1987) Gene, 58:273], and human glucocerebrosidase [Martin et al. (1988) DNA, 7:99] can also be used to provide for secretion in insects.

C.4. Insect cell culture.

Recombinant baculovirus expression vectors have been developed for infection into several insect cells. For example, recombinant baculoviruses have been developed for, inter alia: Aedes aegypti , Autographa californica, Bombyx mori, Drosophila melanogaster, Spodoptera frugiperda, and Trichoplusia ni (P.C.T. Pub. No. WO89/046699; Carbonell et al. (1985) J. Virol., 56:153; Wright (1986) Nature, 321:718, Smith et al. (1983) Mol. Cell. Biol., 3:2156; and see generally, Fraser, et al. (1989) In Vitro Cell. Dev. Biol., 25:225).

Cells and culture media are commercially available for both direct and fusion expression of heterologous polypeptides in a baculovirus/expression, and cell culture technology is generally known to those skilled in the art and described in Summer and Smith (1987).

D. Purification of CMV gH Polypeptides

CMV gH polypeptide is preferably secreted from insect cells and purified from collected media. However, it is also possible to purify CMV gH polypeptides from whole cultures by first lysing cells with a buffered salt solution (e.g. hypotonic PBS) and a detergent usually non-ionic (e.g. 1% NP-40). Typically a disruption buffer may also include 0.5% sodium deoxycholate, 0.1% SDS, and 1mM phenylmethylsulfonyl fluoride (Weir, J.P. and B. Moss, J. Virol. (1985) 56:534-540).

CMV gH polypeptides can be purified from either media or lysate by immunoprecipitation, a technique well known in the art (See for instance, Weir and Moss, (1985)). Murine monoclonal antibody 1G6 (Rasmussen et al., PNAS (1984) 81:876880), described in USSN 367,363, may be used in either immunoprecipitation or affinity column chromatography as described below.

A particular method for purifying CMV gH polypeptide is affinity chromatography using 1G6 monoclonal antibody, described above, which selectively binds to the protein.

The antibody may be covalently coupled to solid supports such as cellulose, polystyrene, polyacrylamide, cross-linked dextran, beaded agarose or controlled pore glass using bifunctional coupling agents that react with functional groups on the support and functional groups (i.e., reactive amino acid side chains) on the antibody molecule. See Scientific Foundations of Clinical Biochemistry, vol. 1, pp.202 at seq, (1978). The resulting monoclonal antibody-bearing solid phase is contacted with disruptates of CMV infected cells or CMV-conditioned media using reducing conditions, pH, ionic strength, temperature (typically physiological), and residence times that permit the gH polypeptide to bind to the immobilized monoclonal antibody. The cells may be disrupted by sonication, lysing or other methods. The solid phase is separated from the disruptate after the incubation and washed with buffer to remove residual unbound disruptate. The protein is eluted from the solid phase by passing an elutant that dissociates hydrogen bonds through the bed. Bases that lower the pH to below about 3 or NaCl solutions above about 2M are commonly used elutants.

Alternatively, a monoclonal antibody prepared by selection of an antibody raised against human CMV gH can be used. Monoclonal antibodies to the glycoprotein may be made by the somatic cell hybridization techniques described initially by Kohler, B. and Milstein, C., Nature (1975) 256:495-497. The procedure involves immunizing a host animal (typically a mouse because of the availability of murine myelomas) with human CMV obtained from infected cultures or with the protein itself. CMV may be grown in human fibroblasts in conventional serum-supplemented liquid growth media such as RPMI 1640 or Dulbecco's minimum essential medium. Virus may be sedimented from culture supernatants by centrifugation.

Antibody-producing cells (e.g., peripheral blood lymphocytes, and splenocytes) are taken from the immunized host and mixed with a suitable tumor fusion partner in a liquid growth medium containing a fusogen such as polyethylene glycol of molecular weight 2000 to 5000. After the fusion the cells are washed to remove residual fusion medium and incubated in a selective growth medium (i.e., a growth medium containing additives to which the parent tumor line is sensitive) such as HAT medium. Only hybrid cells that possess the parent noncancerous cells' ability to survive culture in the selective medium and the parent tumor cells' immortality survive culture in the selective medium. Surviving hybrids may be expanded and their culture media screened for the presence of anti-CMV antibodies by radioimmunoassay (RIA), a micro-neutralization assay that detects inhibition of viral cytopathic effect (CPE) in cell cultures or other assays that detect anti-viral activity (e.g., plaque reduction). Positive cultures may be screened for their ability to recognize and bind to

CMV gH by immunoprecipitating labeled infected cell extracts with the positive cultures and analyzing the precipitate by SDS-PAGE for the presence of a labeled gH component. Hybrids that produce antibody that binds specifically to the protein may be subcloned and growth in vitro or in vivo by known procedures. The antibody may be isolated from the resulting culture medium or body fluid, as the case may be, by conventional procedures for isolating immunoglobulins.

E. Preparation of Vaccines Incorporating CMV gH Polypeptides

The preparation of vaccines which contain an immunogenic polypeptide(s) as an active ingredient(s) is known to one skilled in the art. Typically, such vaccines are prepared as injectables, either as liquid solutions or suspensions; solid forms suitable for solution in, or suspension in, liquid prior to injection may also be prepared. the preparation may also be emulsified, or the polypeptide(s) encapsulated in liposomes. The active immunogenic ingredients are often mixed with excipients which are pharmaceutically acceptable and compatible with the active ingredient. Suitable excipients are, for example, water, saline, dextrose, glycerol, ethanol, or the like and combinations thereof. In addition, if desired, the vaccine may contain minor amounts of auxiliary substances such as wetting or emulsifying agents, pH buffering agents, and/or adjuvants which enhance the effectiveness of the vaccine.

Examples of adjuvants which may be effective include, but are not limited to: aluminum hydroxide, N-acetyl-muramyl-L-threonyl-D-isoglutamine (thr-MDP), N-acetyl-nor-muramyl-L-alanyl-D-isoglutamine (CGP 11637),

referred to as nor-MDP), N-acetylmuramyl-L-alanyl-D-isoglutaminyl-L-alanine-2-(1'-2'-dipalmitoyl-sn-glycero-3-hydroxyphosphoryloxy)-ethylamine (CGP 19835A, referred to as MTP-PE, and RIBI, which contains three components extracted from bacteria, monophosphoryl lipid A, trehalose dimycolate and cell wall skeleton (MPL+TDM+CWS) in a 2% squalene/Tween 80 emulsion. Examples of vaccine formulations comprising oil-in-water emulsions of muramyl peptides and antigen, preferably in a small particle (i.e. sub-micron) emulsion, are disclosed in commonly owned PCT/US90/02954, filed 24 May 1990, and EPA 90.305744.6, filed 25 May 1990, the disclosures of which are incorporated herein by reference.

The effectiveness of an adjuvant may be determined by measuring the induction of antibodies directed against an immunogenic polypeptide containing a CMV gH polypeptide epitope, the antibodies resulting from administration of this polypeptide in vaccines which are also comprised of the various adjuvants.

The polypeptides may be formulated into the vaccine as neutral or salt forms. Pharmaceutically acceptable salts include the acid addition salts (formed with free amino groups of the peptide) and which are formed with inorganic acids such as, for example, hydrochloric or phosphoric acids, or organic acids such as acetic, oxalic, tartaric, maleic, and the like. Salts formed with the free carboxyl groups may also be derived from inorganic bases such as, for example, sodium potassium, ammonium, calcium, or ferric hydroxides, and such organic bases as isopropylamine, trimethylamine, 2-ethylamino ethanol, histidine, procaine, and the like.

The vaccines are conventionally administered parenterally, by injection, for example, either subcutaneously or intramuscularly. Additional

formulations which are suitable for other modes of administration include suppositories and, in some cases, oral formulations. For suppositories, traditional binders and carriers may include, for example, polyalkylene glycols or triglycerides;such suppositories may be formed from mixtures containing the active ingredient in the range of 0.5% to 10%, preferably 1%-2%. Oral formulations include such normally employed excipients as, for example, pharmaceutical grades of mannitol, lactose, starch, magnesium stearate, sodium saccharine, cellulose, magnesium carbonate, and the like. These compositions take the form of solutions, suspensions, tablets, pills, capsules, sustained release formulations or powders and contain 10%-95% of active ingredient, preferably 25%-70%.

The vaccines are administered in a manner compatible with the dosage formulation, and in such amount as will be prophylactically and/or therapeutically effective. The quantity to be administered, which is generally in the range of 5 μg to 250 μg of antigen per dose, depends on the subject to be treated, capacity of the subject's immune system to synthesize antibodies, and the degree of protection desired. Precise amounts of active ingredient required to be administered may depend on the judgment of the practitioner and may be peculiar to each individual.

The vaccine may be given in a single dose schedule, or preferably in a multiple dose schedule. A multiple dose schedule is one in which a primary course of vaccination may be with 1-10 separate doses, followed by other doses given at subsequent time intervals required to maintain and/or reenforce the immune response, for example, at 1-4 months for a second dose, and if needed, a subsequent dose(s) after several months.

The dosage regimen will also, at lest in part, be determined by the need of the individual and be dependent upon the judgment of the practitioner.

In addition, the vaccine containing the polypeptide comprised of an immunogenic CMV gH epitope may be administered in conjunction with other immunoregulatory agents, for example, immune globulins.

Such compositions are useful for treating subjects for CMV. Subjects will generally be mammals, including within that limitation, humans, domestic animals, pets, and sports animals.

III. Examples

Example 1 Expression of Human CMV full-length gH.

In order to investigate the possibility of using the baculovirus system as a means for expressing useful levels of CMV gH, a baculovirus-gH transfer vector encoding full-length gH (gH2) was prepared for use in the baculovirus-insect cell expression system described above. Baculovirus vector pAc373 (FIG. 4, Summers, et al.) was cut with BamHI and the 2495 bp Not1 to Xba1 gH fragment of plasmid pSVgH2 (described in USSN 367,363) was filled and ligated into this BamHI site. The resulting plasmid, designated pACgH2 (see FIG. 5A), encodes a gH construct where transcription is driven by the baculovirus polyhedrin gene promoter. This DNA plasmid was mixed with wildtype baculovirus viral DNA , the mixture was transfected into cells derived from Spodoptera frugiperda (Sf9 cells), and recombinant plaques were isolated and plaque purified. Several recombinant virus clones were used to infect cells, and at four to six days after infection, cell lysates and conditioned media were analyzed by ELISA and Western

blot. No expression of full-length gH (pACgH2) was detected.

Expression of gH in Sf9 cells infected with a recombinant baculovirus containing pACgH2 was also analyzed by radioimmunopercipitation (RIP), using monoclonal IG6 or human sera. No gH-specific bands were detected from RIPs of baculovirus-pACgH2 (full-length gH) infected cells or media.

Example 2 Expression of Truncated Human CMV gH.

A baculovirus-gH transfer vector encoding a fragment of gH, lacking a C-terminal domain, was prepared. Baculovirus vector pAc373 (FIG. 4, Smith, et al., Proc. Natl. Acad. Sci. (1985) 82:8404-8408) was cut with BamHI and the 2178 bp NotI to SalI fragment from pCM6-H6 (See USSN 367,363) was filled and ligated into this BamHI site. The resulting plasmid, designated pACgH6 (ATCC Accession No. 68373, see FIG. 5B) encodes a gH construct where transcription is driven by the baculovirus polyhedrin gene promoter. In this truncated gH segment most of the transmembrane region of gH was deleted by removal of the last 22 amino acids from the C-terminus of gH. The fragment retains only the first three amino acids of the gH transmembrane domain (Leu_{718}-Leu_{719}-Met_{720} - FIG. 2).

The plasmid was mixed with wild-type baculovirus viral DNA, used to cotransfect Spodoptera frugiperda cells and recombinant plaques were isolated and plaque purified. Several recombinant virus clones were used to infect cells, and at four to six days after infection, cell lysates and conditioned media were analyzed by ELISA and Western blot.

For all the pACgH6 containing clones, ELISA analysis showed gH reactivity for the culture media (FIG.

6), indicating that truncated gH was expressed in this system. While ELISA analysis of cell lysates was unable to detect the intracellular presence of truncated gH, RIP analysis was positive for truncated gH, indicating a low but detectable intracellular presence of truncated gH.

Example 3 Time Course for Truncated gH Synthesis During Baculovirus Infection

The time course of truncated gH synthesis during baculovirus infection was analyzed in order to determine the optimal conditions for gH production. As shown in Figure 6, cells were infected at low (0.5 MOI) and high (10.0 MOI) multiplicities with the baculovirus pAcgH6 recombinant, and samples of conditioned media taken from 19 hr to 7 days after infection. For those infections using high protein media (Grace's medium plus 0.33% Yeastolate, 0.33% Lactalbumin Hydrolysate and 10% FCS), the accumulation of gH in the media appeared to plateau after 48 hr. These results indicate that gH synthesis and/or secretion shuts off at 48 hr after infection and that slightly higher levels of gH production are seen in the high MOI infections. Identical infections were also done using EX-CELL 400 (J.R. Scientific) low protein media (FIG. 6). Under these conditions, the maximum level of gH in the media is similar to the infections using high protein media, though synthesis is slower and does not plateau until four days after infection.

Example 4 Radioimmunoprecipitation of Recombinant Truncated gH

Baculovirus recombinants expressing truncated gH have also been analyzed by radioimmunoprecipitation (RIP), using monoclonal IG6 or human sera. A major gH-

specific band of 85 kD and a minor band of 74 kD were detected by RIP from cell lysates and media of S. frugiperda (Sf9) cells infected with a baculovirus-pACgH6 (truncated gH) recombinant. The reason for two gH bands being detected during this 5 hr. pulse is not known, however it is possible that these molecules represent different glycosylation forms.

Expression of full-length gH was also examined using this technique, but was not detectable.

Example 5 Comparison of Expression of Full-Length and Truncated gH in the media of Insect Cells

Insect cell cultures were infected with either pACgH2 (encoding full-length gH) or pACgH6 (encoding truncated gH) and secretion was measured by ELISA assay. Figure 7 shows that while secretion of full-length gH was not detectable, secretion of truncated gH was readily detectable after 24 hours.

Example 6 Comparison of the Level of Expression of Truncated gH in Insect Cells and Mammalian Cells

Stable CHO cell lines transfected with gH expression plasmid pCMAdH6 were isolated (See USSN 367,363). This plasmid encodes the same truncated gH fragment used for pACgH6. These cell lines were subjected to two rounds of MTX amplification and very low levels of gH were detected in conditioned media from these cell lines (CHO line 40 and line 171, 0.1 μM MTX). When the level of extracellular gH from baculovirus infections is compared by ELISA to CHO production, about 10-fold higher levels of gH are produced by the baculovirus recombinants (FIG. 8). The CHO line is in fact expressing some truncated gH because immunoprecipitation experiments show the presence of a

low amount of intracellular truncated gH, but not secreted truncated gH. These results demonstrate that the baculovirus system, surprisingly, is more at least 10 times more efficient than CHO cells for the production of extracellular gH.

CLAIMS

What is Claimed:

1. A baculovirus-gH transfer vector consisting of a plasmid, which plasmid comprises DNA which is substantially homologous to baculovirus DNA, and a segment of DNA which encodes a human cytomegalovirus (CMV) glycoprotein H (gH) polypeptide.

2. The vector of claim 1 wherein said human CMV gH polypeptide is a truncated analog of native human CMV gH.

3. The vector of claim 2 wherein said analog is lacking all or a portion of a transmembrane binding domain which is present in native human CMV gH.

4. The vector of claim 3 wherein at least about 10 contiguous amino acids from residue 709 through residue 742 in Figure 2 are not present in said analog.

5. A baculovirus vector comprising DNA which encodes a human CMV gH polypeptide in which expression of said human CMV gH polypeptide is regulated by a baculovirus regulatory element.

6. The vector of claim 5 wherein said human CMV gH polypeptide is a truncated analog of native human CMV gH.

7. The vector of claim 6 wherein said analog is lacking all or a portion of a transmembrane binding domain which is present in native human CMV gH.

8. The vector of claim 7 wherein at least about 10 contiguous amino acids from residue 709 through residue 742 in Figure 2 are not present in said analog.

9. The baculovirus vector of claim 5 in which said element is the baculovirus polyhedrin gene promoter.

10. An insect cell comprising DNA which encodes a human CMV gH polypeptide and which is capable of expressing said human CMV gH polypeptide.

11. The cell of claim 10, said cell being derived from "Spodoptera frugiperda".

12. The cell of claim 10 in which said human CMV gH polypeptide encoding DNA has been introduced by infection with a recombinant baculovirus.

13. A method for producing a human CMV gH polypeptide, said method comprising:

a) providing the insect cell of claim 10, said DNA being transcriptionally regulated by a promoter derived from a baculovirus;

b) incubating the cell under conditions in which said human CMV gH polypeptide is expressed; and

c) recovering said expressed human CMV gH polypeptide.

14. The method of claim 13 in which a truncated analog of native human CMV gH is produced.

15. The method of claim 13 wherein said promoter is baculovirus polyhedrin promoter.

16. The method of claim 15 in which the baculovirus is "Autographa californica".

17. The method of claim 13 in which said insect cell host cell is derived from "Spodoptera frugiperda".

18. The method of claim 13 in which the cytomegalovirus is strain Towne.

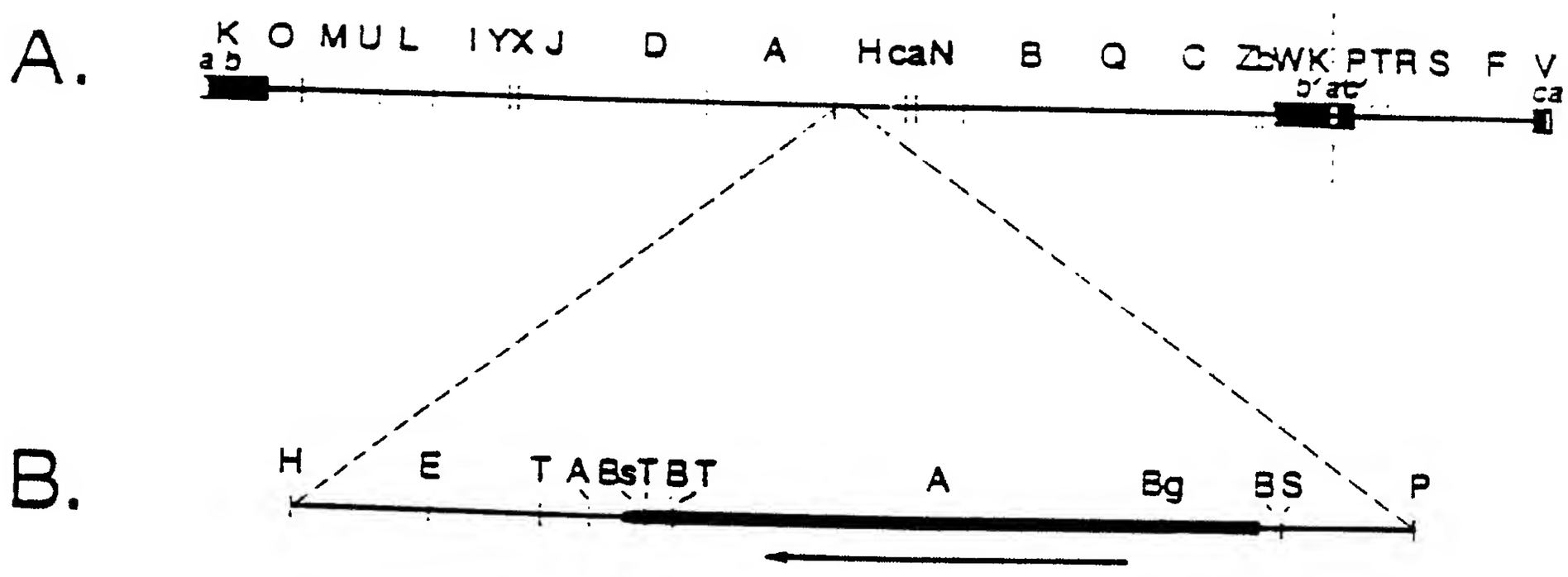
19. The method of claim 13 in which the cytomegalovirus is AD-169.

20. A human CMV gH polypeptide said polypeptide being glycosylated by means of secretion from the cell of claim 10.

21. A method for treating a subject for CMV infection comprising administering an effective amount of human CMV gH polypeptide in a pharmaceutically acceptable carrier.

22. A composition comprising the CMV gH polypeptide of claim 20 in a pharmaceutically acceptable carrier.

Figure 1.


A.
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a b
b' a'
ca
B.
H E T A Bs T B T A Bg B S P

Figure 2

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                                                                                                                          3048
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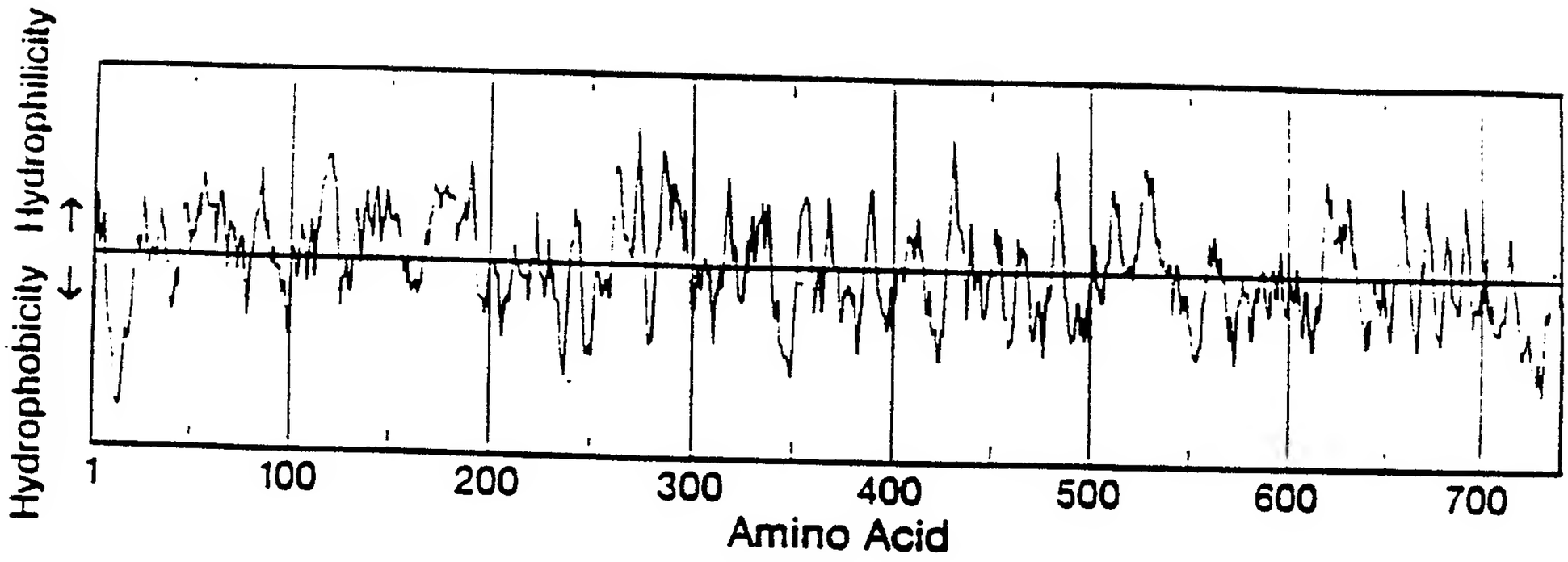
Figure 3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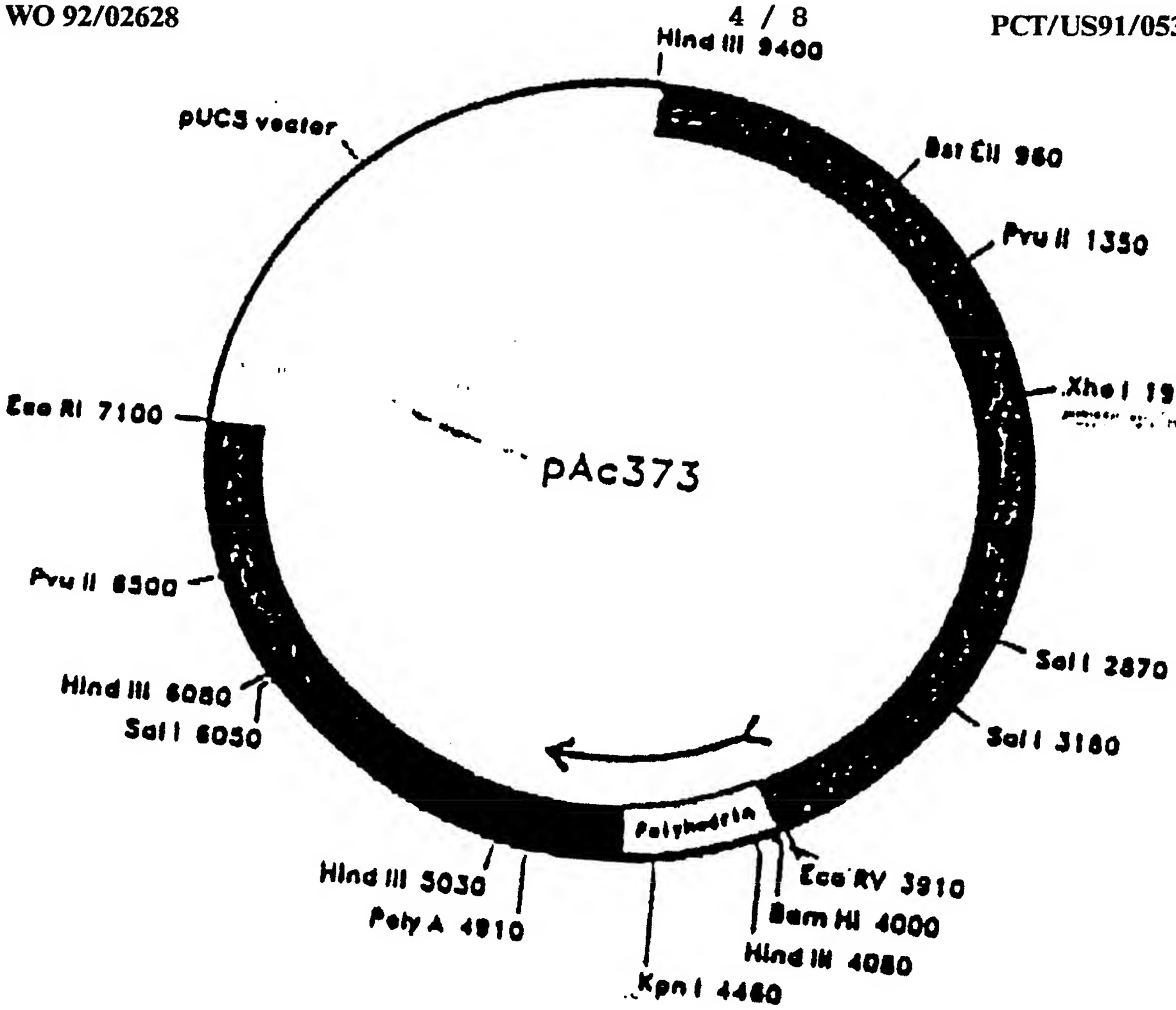

+1

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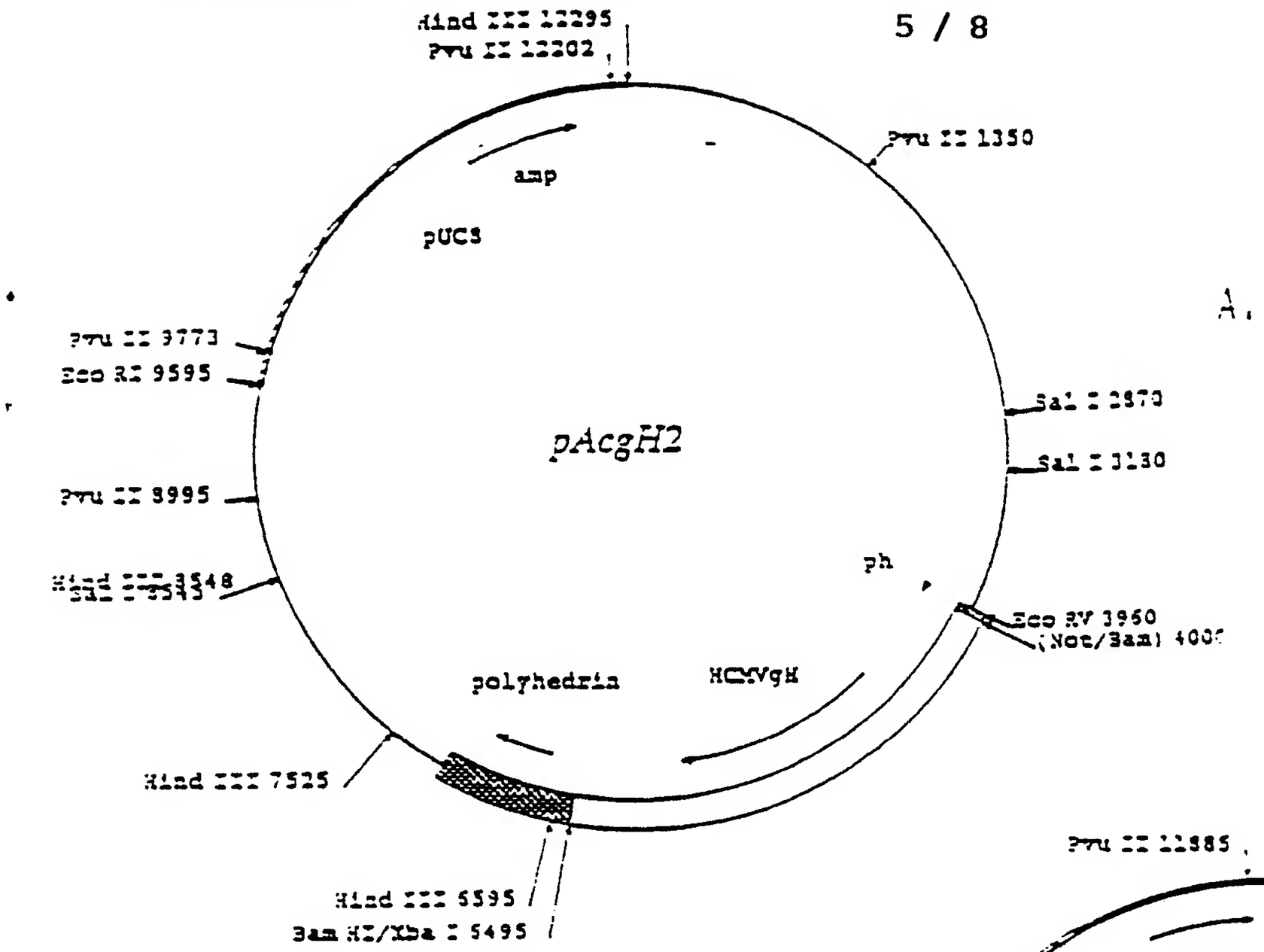
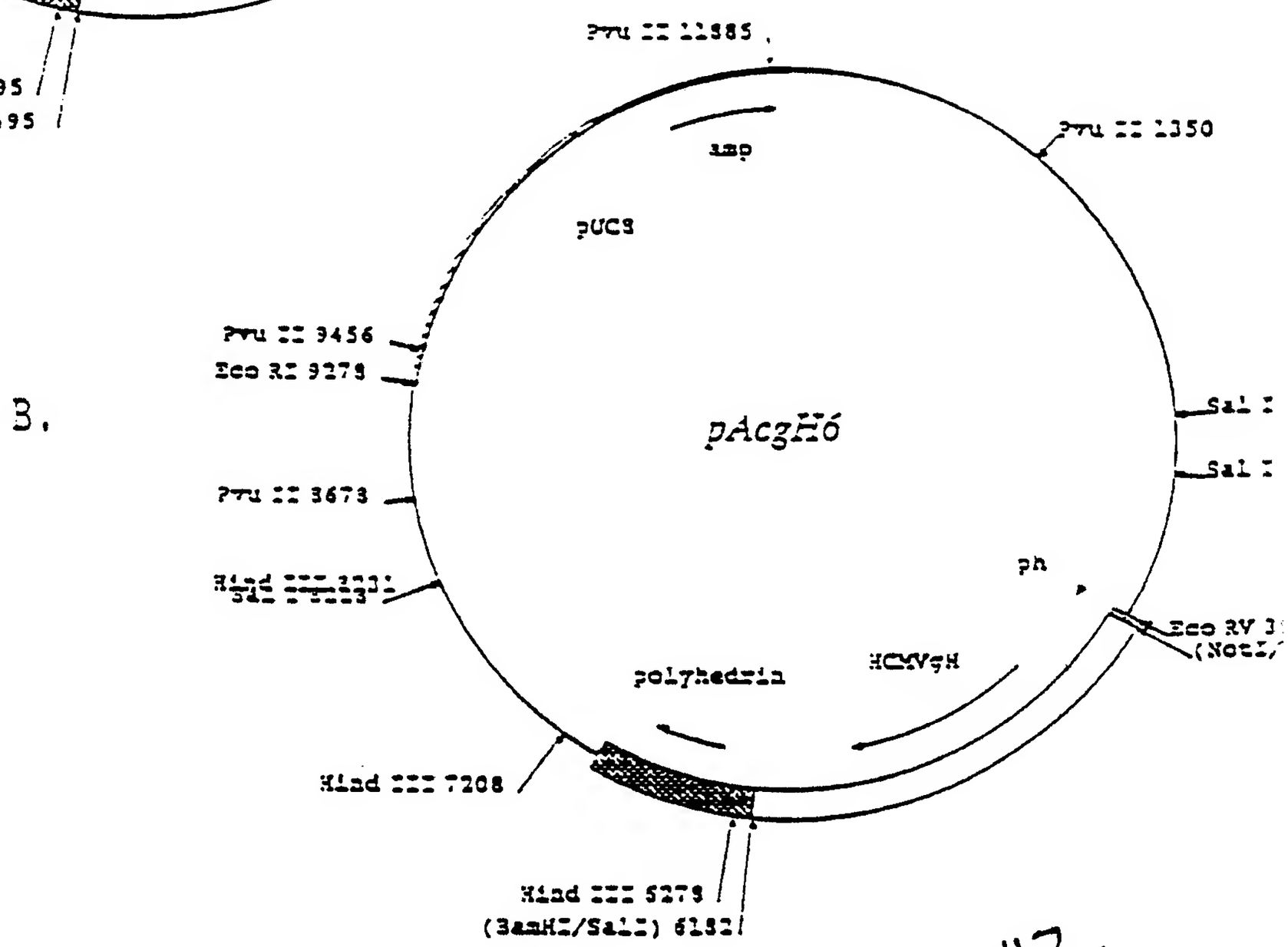
Figure 4



A.



B.

Figure 5. gH-baculovirus transfer vectors.

Plasmid pAcgH2 (A) encodes the full-length gH gene, including 9 bp of 5'-untranslated sequences and 251 bp of 3'-untranslated sequences (including a polyA addition site) isolated as a 2495 kb NotI to XbaI fragment from ~~pCMV-HA~~ pSVgH2. Plasmid pAcgH6 (B) encodes a C-terminal truncated gH gene, with 9 bp of 5'-untranslated sequences and lacking th transmembrane and cytoplasmic domains, isolated as a NotI to SalI fragment from pCM6-H6. Both gH fragments were blunt-ligated into the BamHI site of baculovirus transfer vector pAc373 such that gH transcription is driven by the baculovirus polyhedrin promoter.

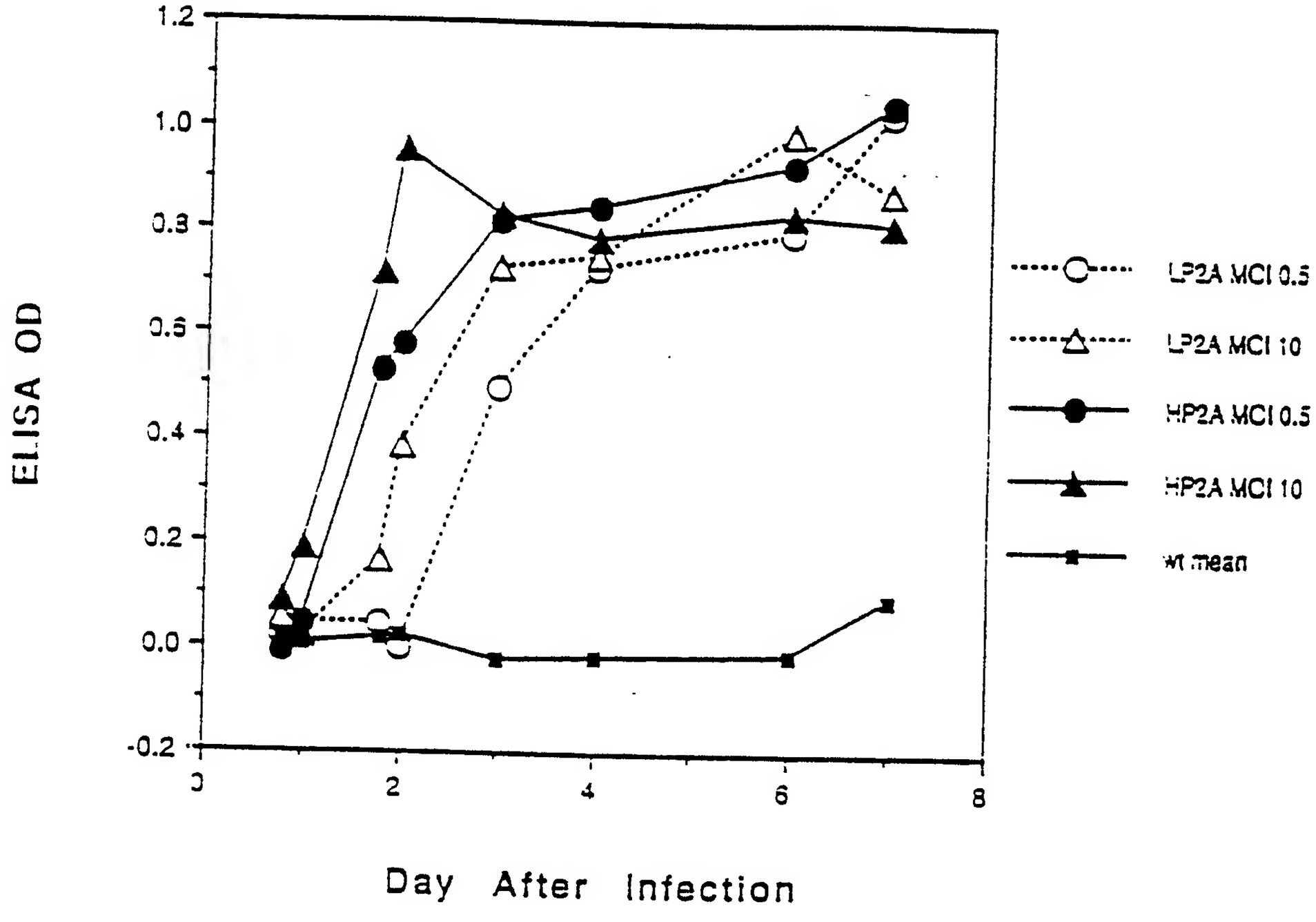


Figure 6 Secretion of gH from cells infected with a baculovirus recombinant expressing truncated gH.

S. frugiperda cells were infected with baculovirus-gH recombinant virus clone 2A, which was generated by recombination of wild type baculovirus with pACgH6 (see July, 1989 report). The cells were infected either at a multipicity of infection (MOI) of 10 or 0.5. Two types of media were also tested: Grace's medium plus 0.33% Yeastolate, 0.33% Lactalbumin Hydrolysate and 10% FCS, high protein media (HP), or EX-CELL 400, low protein media (LP). Samples of media were taken as indicated and analyzed by the gH ELISA. Identical infections were also done with wild type virus (wt) which does not encode gH. The mean of the wt curves is shown because these controls were identical for both multipicities and medias.

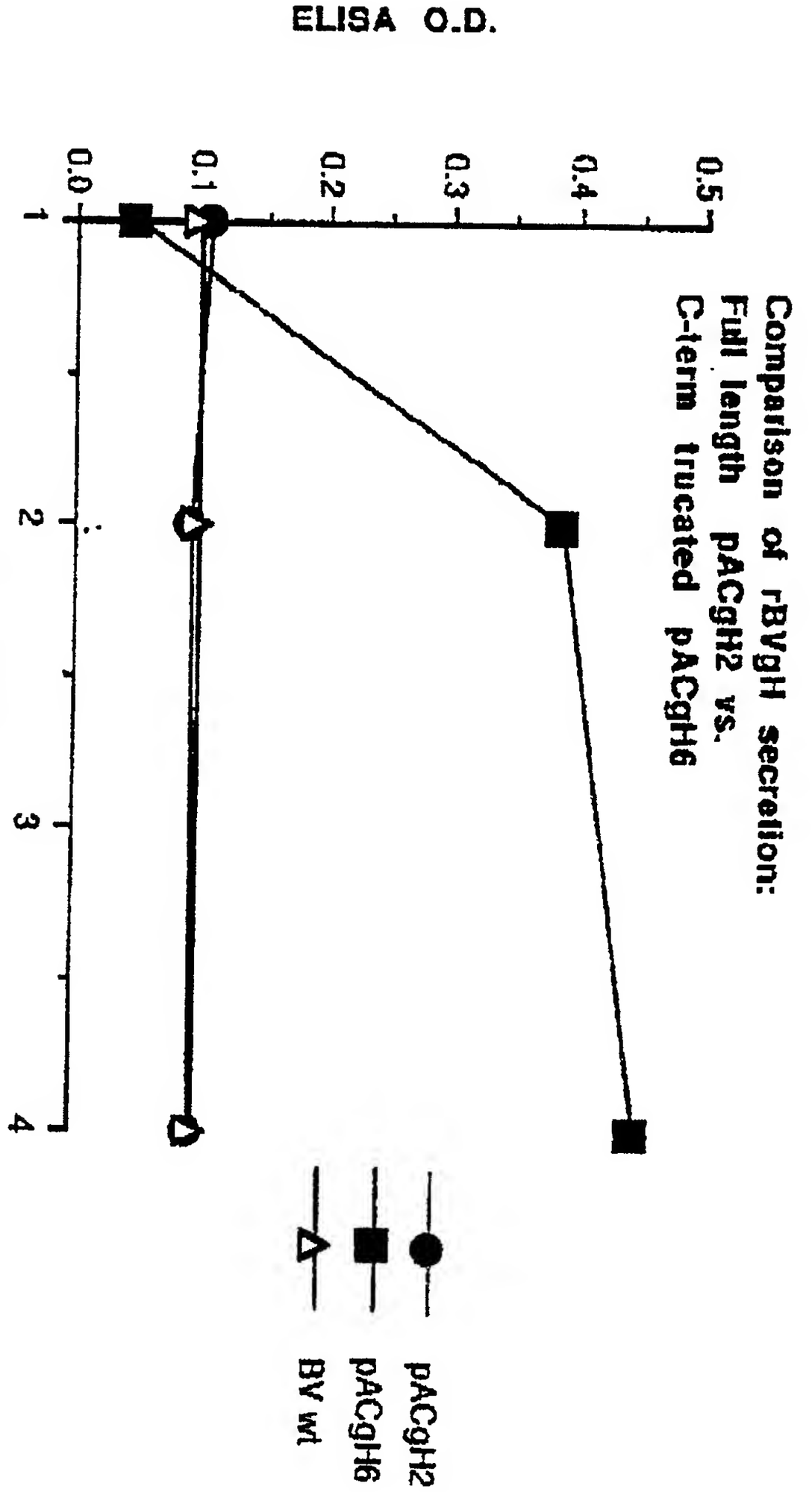

ELISA O.D.
0.0
0.1
0.2
0.3
0.4
0.5
1
2
3
4
Days P.I.
Comparison of rBVgH secretion:
Full length pACgH2 vs.
C-term trucated pACgH6
BV wt
pACgH6
pACgH2

Figure 7

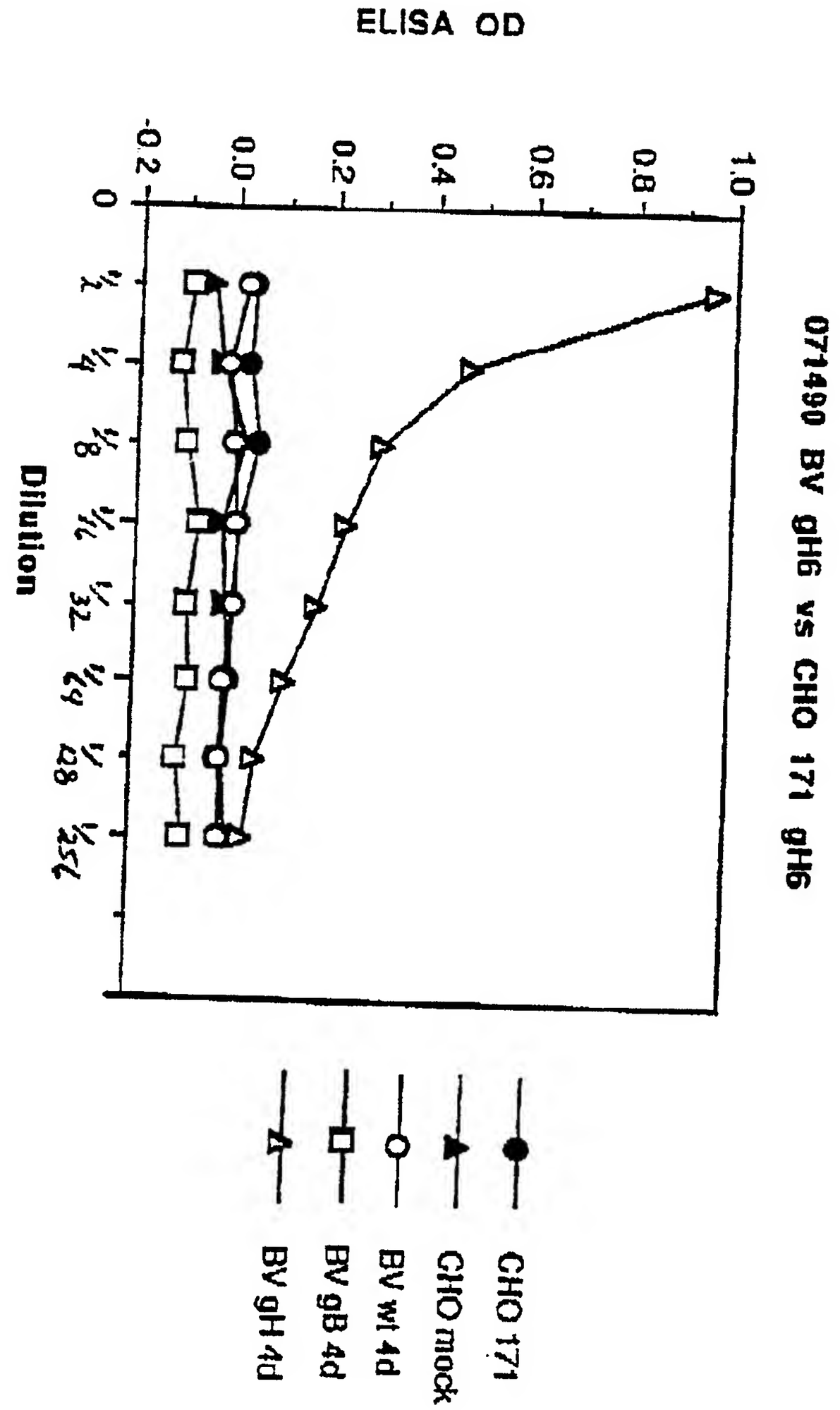

ELISA OD
0
0.2
0.4
0.6
0.8
1.0
Dilution
1/25
1/50
1/100
1/200
1/400
1/800
1/1600
1/3200
1/6400
1/12800
071490 BV GH6 vs CHO 171 GH6
CHO 171
CHO mock
BV wt 4d
BV GB 4d
BV GH 4d

Figure 8

INTERNATIONAL SEARCH REPORT

International Application No. PCT/US91/05324

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [6]

According to International Patent Classification (IPC) or to both National Classification and IPC

U.S.: 435/69.1,240.27,320;935/22,34
IPC(5): C12P 21/06, C12N 5/00, 15/00,

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
US	435/69.1,240.27,320; 935/22,34

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [8]

APS, Dialog, CAS

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category [10]	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
Y	Cell, Volume 30, issued October 1982, J.K. Rose et al., "Expression from Cloned cDNA of Cell-Surface Secreted Forms of the Glycoprotein of Vesicular Stomatitis Virus in Eucaryotic Cells", pages 753-762, see entire document.	2-4, 6-8,14
Y	Virology, Volume 169, issued 1989, C. Pachl et al., "The Human Cytomegalovirus Strain Towne Glycoprotein H Gene Encodes Glycoprotein p 86", pages 418-426, see entire document.	1,5,9-13, 15-19
Y	Journal of General Virology, Volume 71, issued April 1990, D.E. Wells et al., "Structural and Immunological Characterization of Human Cytomegalovirus pg 55-116 (gB) expressed in insect cell", pages 873-880, see entire document.	1,5,9-13,15-19,22
Y	US,A 4,745,051 (Smith et al) 17 May 1988, see entire document.	1,5,9-13, 15-19,20

* Special categories of cited documents: [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
20 November 1991	08 JAN 1992
International Searching Authority	**Signature of Authorized Officer**
ISA/US	Suzanne Ziska

Form PCT/ISA/210 (second sheet) (Rev.11-87)